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Monkeypox-Induced Immunity and Failure of Childhood Smallpox Vaccination To Provide Complete Protection▽

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Following the U.S. monkeypox outbreak of 2003, blood specimens and clinical and epidemiologic data were collected from cases, defined by standard definition, and household contacts of cases to evaluate the role of preexisting (smallpox vaccine-derived) and acquired immunity in susceptibility to monkeypox disease and clinical outcomes. Orthopoxvirus-specific immunoglobulin G (IgG), IgM, CD4, CD8, and B-cell responses were measured at ~7 to 14 weeks and 1 year postexposure. Associations between immune responses, smallpox vaccination, and epidemiologic and clinical data were assessed. Participants were categorized into four groups: (i) vaccinated cases, (ii) unvaccinated cases, (iii) vaccinated contacts, and (iv) unvaccinated contacts. Cases, regardless of vaccination status, were positive for orthopoxvirus-specific IgM, IgG, CD4, CD8, and B-cell responses. Antiorthopoxvirus immune responses consistent with infection were observed in some contacts who did not develop monkeypox. Vaccinated contacts maintained low levels of antiorthopoxvirus IgG, CD4, and B-cell responses, with most lacking IgM or CD8 responses. Preexisting immunity, assessed by high antiorthopoxvirus IgG levels and childhood smallpox vaccination, was associated (in a nonsignificant manner) with mild disease. Vaccination failed to provide complete protection against human monkeypox. Previously vaccinated monkeypox cases manifested antiorthopoxvirus IgM and changes in antiorthopoxvirus IgG, CD4, CD8, or B-cell responses as markers of recent infection. Antiorthopoxvirus IgM and CD8 responses occurred most frequently in monkeypox cases (vaccinated and unvaccinated), with IgG, CD4, and memory B-cell responses indicative of vaccine-derived immunity. Immune markers provided evidence of asymptomatic infections in some vaccinated, as well as unvaccinated, individuals.

In 2003, a zoonotic outbreak of human monkeypox occurred in North America. The outbreak was the first time that this virus caused human disease outside Africa. Subsequent investigation confirmed that the source of the outbreak was an imported consignment of African mammals from the West African nation of Ghana. Subsequently, the virus moved to captive native North American species, including *Cynomys* species (prairie dogs) (10, 21, 23). Human cases were infected through contact with infected animals, principally prairie dogs (19, 23). Viral isolates associated with the outbreak were genetically characterized as West African variants of monkeypox distinct from Central African viruses (20). During the outbreak, and in a follow-up study, specimens from suspected cases and their contacts were submitted to the Centers for Disease Control and Prevention (CDC) for laboratory testing. Laboratory testing included virological testing (culture and PCR) of presumptive virus-containing specimens for case confirmation and analysis of immunological markers from blood or serum samples. Immunological analysis included serologic testing for orthopoxvirus (OPX)-specific antibodies (immunoglobulin G [IgG] and IgM) and virus-specific cellular (CD4, CD8, and B-cell) immunity evaluation in cases and contacts.

In this study, we evaluated human monkeypox cases and household contacts of monkeypox-infected animals and report the characterization of the convalescent and long-term memory (1-year) immune response to monkeypox infection during this outbreak. Correlations between immunological markers, smallpox vaccination status, and infection outcomes are evaluated, as well as memory immune responses in previously vaccinated individuals within this cohort.

MATERIALS AND METHODS

Specimens. Response to the 2003 U.S. monkeypox outbreak involved the collection of information related to lab results, exposure characteristics, clinical features of illness, and other epidemiologic features. Teams of clinicians, epidemiologists, and microbiologists from the CDC and the six affected states collected information and samples from cases and contacts of cases. Patient specimens were sent to the CDC as a part of the outbreak investigation, and confirmation of suspect cases was based on identification of virus in tissue-derived specimens (21). As an extension of the outbreak investigation, a follow-up, household-based, Institutional Review Board-approved, case control study was initiated to assess exposures, clinical markers of disease severity, and immune responses at 7 to 14 weeks (convalescent) and at 1 year postexposure. Specimens were collected at convalescent and 1-year time points, and clinical and epidemiologic information was collected with a standardized questionnaire. A total of 92 persons enrolled in the study. Eighteen of those enrolled were excluded from the analysis due to unknown vaccination status or unwillingness to provide biological samples. A total of 72 individuals were considered in these analyses. Enrolled individuals were categorized into four groups: cases with previous smallpox vaccination, cases without previous smallpox vaccination, con-

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TABLE 1. Variables used in analysis

Variable	Definition or division
Severity	Mild: ≤25 lesions at ht of illness
	Moderate to severe: >25 lesions at ht of illness
Ig response (within 4 wk post-rash)[a,e]	
IgG	Low (<0.62)
	High (≥0.62)
IgM	Low (<0.1533)
	High (≥0.1533)
Age	<31 yr[b]
	≥31 yr
T-cell response[c,e]	
CD4	Low (<0.04)
	High (≥0.04)
CD8	Low (<0.21)
	High (≥0.21)
Memory B cells[d,e]	Low (<0.27954)
	High (≥0.27954)

[a] OD − COV, OD at 450 nm minus the COV as indicated in Materials and Methods.

[b] Approximate age cutoff for determination of individuals who were likely to have been previously vaccinated against smallpox.

[c] Frequency of OPX-specific T cells; determined as a function of the percentage of cells expressing IFN-γ in response to OPX antigen stimulation compared to the total T-cell (CD4 or CD8) population.

[d] Average OPX-specific IgG production B-cell spot divided by the total IgG spot count (all IgG-producing cells) equals the frequency.

[e] High and low categories were created using the median value of the mild-disease group.

tacts with previous smallpox vaccination, and contacts without previous smallpox vaccination. Standardized case definitions were used to assign confirmed and probable case status (http://www.cdc.gov/ncidod/monkeypox/casedefinition.htm), and these individuals are analyzed as "cases" within this paper. All participants provided informed consent, and Institutional Review Board approval was obtained through CDC and Emory University.

Variables. Variables used to compare illness included disease severity, humoral and cellular immunity, age, and smallpox vaccination history (Table 1). Information about number of lesions (at height of illness) was used as a proxy to define disease severity. A moderate to severe case of monkeypox was defined as a case with >25 lesions on the body at the height of the illness. A mild case was someone with ≤25 lesions.

The IgG and IgM level variable was designed by partitioning IgG and IgM OD − COV (optical density − cutoff value) levels (as indicated under "ELISA") into "high" and "low" response categories based on median values of the mild disease group for samples collected within 4 weeks after rash onset (see Fig. 4). Laboratory results relating to cellular immunity, CD4, CD8, and memory B cells were also dichotomized into "high" and "low" response variables based on median levels in the mild disease group for samples collected at convalescence (see Fig. 4). Age was dichotomized into <31 years of age and ≥31 years of age, to easily distinguish persons who were young enough to have been born after the cessation of routine smallpox vaccinations (in the United States) from those who could have received childhood vaccinations.

Immune analysis. (i) ELISA. Serum samples from suspect cases were tested by use of an IgM capture assay and IgG enzyme-linked immunosorbent assay (ELISA) to detect specific antibodies (anti-OPX) as previously described (16). In order to compare absolute OD values and minimize variability, serum samples used in comparative analyses were run in parallel using a quality-controlled lot of reagents, on the same day. Both known positive- and negative-control sera were used for assay standardization and quality control. COVs were determined from the average absorbance of five negative-control sera plus 3 standard deviations and used as the baseline for the limit of detection in each assay. Reported values were determined as the raw absorbance minus the COV for each individual assay run (OD − COV) in order to normalize and compare assay-to-assay results. Vaccinia virus antigen was passaged from DryVax vaccine four times in BSC-40 cells, and this stock was used for ELISA, to detect OPX-specific reactivity. Passaged virus was harvested and purified as described previously (16).

(ii) Cellular immunity. ***(a) Intracellular cytokine staining assay.*** Peripheral blood cells were collected and used to measure OPX-specific T-cell activity as previously described (2). Intracellular cytokine levels were determined following in vitro antigen stimulation (vaccinia virus strain WR) of fresh peripheral blood mononuclear cells (PBMC). Briefly, approximately 1×10^6 PBMC were stimulated in 5-ml polypropylene tubes in RPMI containing 10% fetal bovine serum, anti-human CD28, and anti-human CD49d (1 μg per ml each; Pharmingen, Inc. San Diego, CA) in 100 μl. Approximately 1×10^7 PFU of vaccinia virus strain WR (multiplicity of infection of 10) was added in a volume of 100 μl. After 12 h of incubation at 37°C, 900 μl of RPMI containing 10% fetal bovine serum and monensin (10 μg/ml) was added and cells were cultured for an additional 3 h at 37°C at an angle of 5 degrees. Cells were surface stained with fluorochrome-conjugated antibodies to CD8 (clone SK1; Becton Dickinson) at 8° to 10°C for 30 min, washed once with cold phosphate-buffered saline (PBS) containing 2% fetal bovine serum, and fixed and permeabilized with Cytofix/Cytoperm solution (Pharmingen, Inc.). Cells were then incubated with fluorochrome-conjugated antibodies to human CD3 (clone UCHT1; Beckman Coulter) and gamma interferon (IFN-γ) (clone B27; Pharmingen) in Perm wash solution (Pharmingen) for 30 min at 4°C. Cells were washed twice with Perm wash and once with plain PBS and resuspended in 1% formalin in PBS. Approximately 200,000 lymphocytes were acquired on the FACScalibur and analyzed using FloJo software (Treestar Inc., San Carlos, CA). Lymphocytes were identified based on their scatter pattern, and $CD3^+$ $CD8^-$ cells were considered CD4-positive T cells and $CD3^+$ $CD8^+$ cells were considered CD8-positive T cells. Using this assay, we could detect vaccinia virus-specific CD4 and CD8 T cells at concentrations as low as 0.01% of the respective total cells. In (unvaccinated) contacts, the frequencies of vaccinia-virus specific CD4 and CD8 T cells were below 0.01%.

(b) Memory B-cell assay. Peripheral blood cells were collected and used to measure OPX-specific B-cell activity as previously described (3, 4). In brief, PBMC were plated in 24-well dishes at 5×10^5 cells/well in R-10 supplemented with a mix of polyclonal mitogens: 1/100,000 pokeweed mitogen extract (made at Emory University), 6 μg/ml phosphothioated CpG ODN-2006, and 1/10,000 *Staphylococcus aureus* Cowan (Sigma). Three to nine wells were cultured per individual. Cells were cultured for 5 to 6 days at 37°C and 6 to 8% CO_2. In preparation for the ELISPOT assay, 96-well filter plates (Millipore; MAHA N4510) were coated overnight with vaccinia virus antigen (see below) diluted 1:5 in PBS. Uninfected HeLa cell lysate prepared in an identical manner was used as a negative-control antigen. To detect all IgG-secreting cells, a separate plate was coated with 10-μg/ml goat anti-human Ig (Caltag). Plates were washed and blocked with RPMI 1640 plus 1% bovine serum albumin (fraction V; Sigma) for 2 to 4 h at 37°C prior to use.

Cultured PBMC were washed thoroughly, plated onto ELISPOT plates, and incubated for 5 to 6 h at 37°C. Detection reagents were 1-μg/ml mouse anti-human pan-IgG Fc biotin-conjugated antibody (Hybridoma Reagent Laboratory no. HP6043B) in PBS plus 0.05% Tween 20 plus 1% fetal calf serum, followed by 5-μg/ml-horseradish peroxidase-conjugated avidin D (Vector Laboratories), and developed using 3-amino-9-ethyl-carbozole (Sigma). ELISPOT plates of convalescent-period samples were counted by eye, while assays at the 1-year time point were machine counted with a C.T.L. plate reader and accompanying imaging software (Cellular Technology, Ltd., Cleveland, OH), resulting in different absolute values. Data are represented as the frequency of vaccinia virus-specific B cells as a percentage of the total IgG^+ memory B cells in PBMC.

Vaccinia virus antigen preparation was made by growing vaccinia virus strain WR on HeLa cells, infecting them at a multiplicity of infection of 0.5. Cells were harvested at 60 h, and virus was isolated by rapid freeze-thawing of the cell pellet three times in a volume of 2.3 ml RPMI plus 1% fetal calf serum. Cell debris was removed by centrifugation. Clarified supernatant was frozen at −80°C as virus stock ($\sim 2 \times 10^8$ PFU/ml). Vaccinia virus antigen was then UV inactivated in the presence of trioxsalen-psoralen (4′-aminomethyltrioxsalen HCl; Calbiochem).

RESULTS

Participants' demographics. Seventy-two individuals were included for analysis of immune responses at convalescent and 1-year time points (Fig. 1 to 5). Of these, 27 were designated as cases and 45 were designated as contacts. For this analysis, participants were categorized into four groups based on case status (case or contact) and vaccination history (vaccinated or

TABLE 2. Characteristics of cases with mild versus moderate to severe monkeypox disease

Characteristic	No. (%) of:		*P* value
	Moderate to severe cases (n = 18)	Mild cases (n = 12)	
Previous smallpox vaccination			
Yes	1 (9.09)	5 (27.78)	
No	10 (90.91)	13 (72.22)	0.3623[b]
Missing	1		
Sex[a]			
Male	6 (50.00)	7 (38.89)	
Female	6 (50.00)	11 (61.11)	0.5474
Age (yr)			
<31	9 (75.00)	10 (55.56)	
≥31	3 (25.00)	8 (44.44)	0.4425[b]
IgM (≤4 wk post-rash)			
Low (<0.1533)	4 (50.00)	6 (46.15)	
High (≥0.1533)	4 (50.00)	7 (53.85)	1[b]
Missing	4		5
IgG (≤4 wk post-rash)			
Low (<0.62)	6 (75.00)	6 (46.15)	
High (≥0.62)	2 (25.00)	7 (53.85)	0.3666[b]
Missing	4		5
CD4			
Low (<0.04)	4 (50.00)	3 (33.33)	
High (≥0.04)	4 (50.00)	6 (66.67)	0.6372[b]
Missing	4		9
CD8			
Low (<0.21)	2 (25.00)	4 (44.44)	
High (≥0.21)	6 (75.00)	5 (55.56)	0.6199[b]
Missing	4		9
Memory B cells			
Low (<0.27954)	6 (66.67)	5 (41.67)	
High (≥0.27954)	3 (33.33)	7 (58.33)	0.387[b]
Missing	3		6

[a] Chi-square test result was 0.3620 (1 df).
[b] Exact test *P* value.

unvaccinated). Participants per group were 6 cases with previous smallpox vaccination, 21 cases without previous smallpox vaccination, 27 contacts with previous smallpox vaccination, and 18 contacts without previous smallpox vaccination. Each category was assessed for immune response to OPX (vaccinia virus antigen) and compared to one another at convalescent and 1-year time points.

For assessment of factors correlating with disease severity 30 cases were considered, including the 27 used in immune analysis as well as three additional participants, one of whom was missing vaccination status and the other two of whom were lacking convalescent-period and 1-year samples (Table 2).

Humoral immunity. Convalescent-period (7 to 14 weeks) and 1-year postexposure serum samples were evaluated for the presence of OPX-specific antibody. ELISA was performed to detect IgM and IgG antibodies reactive against OPX antigen (Fig. 1 and 2). Convalescent-period anti-OPX IgM responses were observed in both vaccinated (three of four) and unvaccinated (13 of 16) cases and were maintained at 1 year post-rash onset (1 of 5 and 5 of 11, respectively) at (greatly) reduced levels (Fig. 1a and c). Unvaccinated cases had the highest median convalescent-period IgM values. Of the vaccinated cases with IgM responses, one of the three manifested values in the range of the unvaccinated cases and the other two demonstrated low but detectable IgM responses (Fig. 1a). With rare exceptions, vaccinated contacts did not develop anti-OPX IgM responses (Fig. 1b; Table 3). No unvaccinated contacts had detectable anti-OPX IgM responses above the equivocal range (OD − COV, >0.04).

Anti-OPX IgG levels were observed in most cases at convalescent (18 of 20) and 1-year (16 of 16) time points (Fig. 2a and c). Higher mean anti-OPX IgG levels were observed in previously vaccinated cases (Fig. 2a, median bars) than in vaccinated contacts at convalescent and 1-year sampling times (Fig. 2c, median bars). Vaccinated and unvaccinated cases demonstrated, on average, a 0.4- to 0.5-OD − COV decline in antibody levels from convalescent to 1-year memory sampling; this represents ~20% loss of anti-OPX antibody recognition in vaccinated cases and ~40% loss of anti-OPX antibody recognition in unvaccinated cases from convalescent to 1-year memory time points. The majority of contacts, regardless of vaccination status, demonstrated no change in titer from convalescent to 1-year sampling. Previously vaccinated contacts maintained substantial residual anti-OPX IgG levels over the 1-year period of observation (Fig. 2b). Unvaccinated contacts had no measurable anti-OPX IgG with the exception of three individuals (Fig. 2d; Table 3).

Cellular immunity. Analyses of OPX-specific IFN-γ-producing CD4 and CD8 T-cell responses were performed using an intracellular cytokine staining assay (Fig. 3). OPX-specific CD4 reactivity was observed in vaccinated and unvaccinated cases with similar geometric mean levels both at convalescent and at 1-year time points (Fig. 3a and b, mean bars) with two vaccinated cases showing increases in CD4 reactivity at 1 year (Fig. 3a and b). OPX-specific CD4 responses were observed in contacts with previous vaccination (3 of 14 at convalescence and 2 of 6 at 1 year), but at a lower geometric mean level, and less frequently, with respect to those seen in confirmed cases. No unvaccinated contacts had detectable levels of OPX-specific CD4 T cells at convalescent or 1-year sampling. In cases, geometric mean OPX CD4 reactivities and distribution of responses at convalescent and 1-year samplings were similar. Two vaccinated cases showed a log increase in CD4 counts at 1 year (Fig. 3a versus 3b), while all other cases with paired samplings showed stable responses within 0.5 logs from convalescence to 1 year.

OPX-specific CD8 responses were observed at convalescent and 1-year time points in both vaccinated (three of five at convalescence and five of five at 1 year) and unvaccinated (9 of 12 at convalescent and 7 of 7 at 1 year) cases, with higher geometric mean levels seen in those who were unvaccinated (Fig. 3c and d). Using the IFN-γ intracellular cytokine measurements, OPX-specific CD8 responses were below the level of detection (less than 0.01% of total CD8 cells) in all contacts, with the exception of two vaccinated contacts with elevated 1-year levels (Fig. 3d; Table 3).

Memory B cells were detected in 17 out of 19 cases tested at convalescence including 13 unvaccinated cases. At 1 year, 13

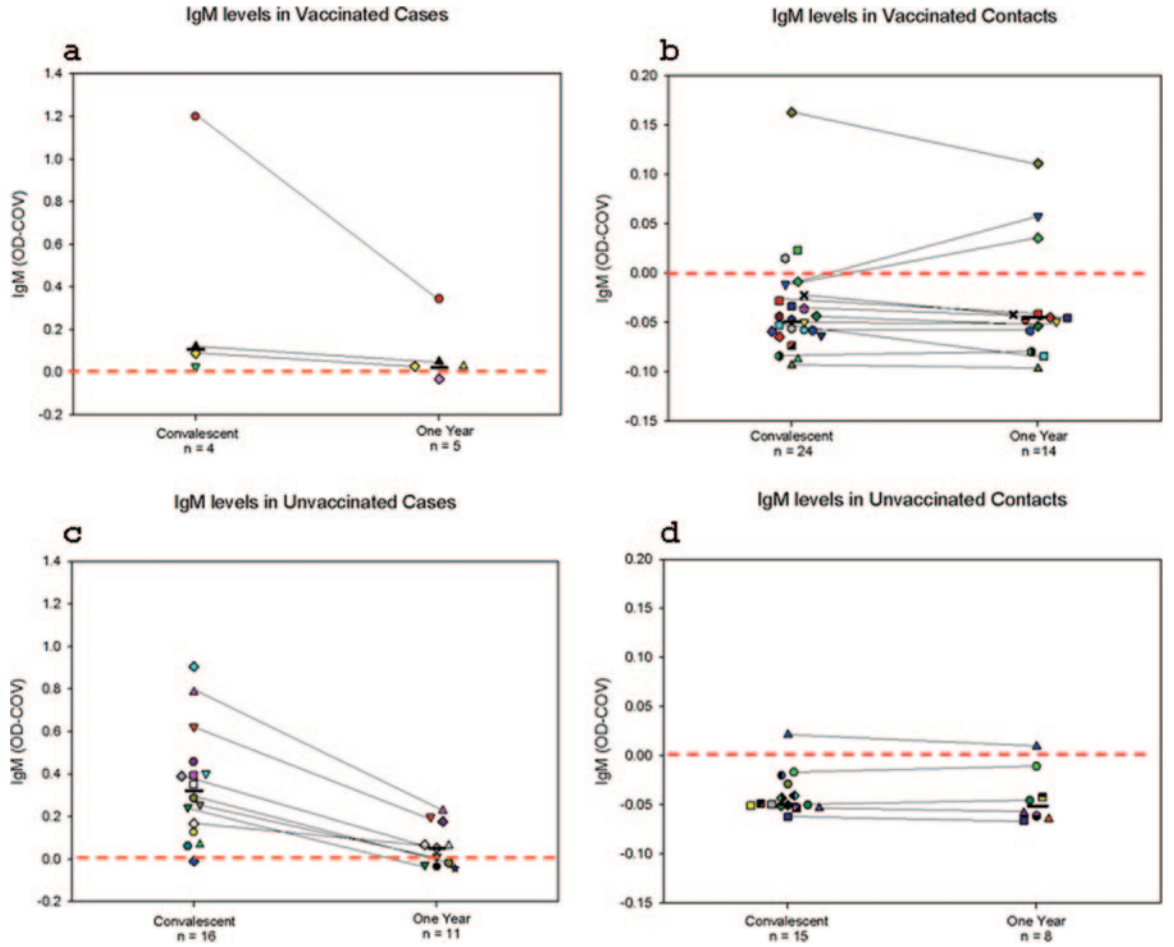

FIG. 1. ELISA of anti-OPX IgM responses in convalescent and 1-year sampling of all cases and contacts based on vaccination status. Results are presented as OD − COV where values above zero are considered positive. IgM values of 0 to 0.04 are considered equivocal. Symbols represent individuals involved in the study and remain consistent throughout the figures.

out of 13 cases tested positive for memory B cells, irrespective of vaccination status. At convalescence, the highest median OPX-specific memory B-cell responses were observed in vaccinated cases, with lower geometric mean responses observed in unvaccinated cases as well as previously vaccinated contacts (Fig. 4). Among vaccinated cases, three had decreased OPX-specific B-cell counts at 1 year, while one had increased counts at 1 year. Among unvaccinated cases, OPX-specific B-cell counts (geometric mean and individual) remained similar from convalescence to 1 year. Vaccinated contacts show a reduction in B-cell counts for one individual from convalescence to 1 year, while in one instance, counts rose significantly (Fig. 4a and b; Table 3). No significant OPX-specific B-cell responses were seen in unvaccinated contacts at convalescence or 1-year sampling with the exception of the aforementioned 35-year-old who also exhibited detectable IgG at 1 year (Fig. 4b; Table 3). No convalescent-period cellular sample was submitted for this individual.

Contacts with signs of OPX immune stimulation. As indicated in the previous sections, a number of individuals, classified as contacts, were noted to have convalescent-period and/or memory OPX immune responses in the absence of any discernible rash illness, although other clinical symptoms of viral infection may have been present (Table 3). Two contacts vaccinated against smallpox as children manifested positive anti-OPX IgM responses, one positive at both time points tested and one negative at convalescence and positive at 1 year (Fig. 1b; Table 3, ages 49 and 60, respectively). Another vaccinated contact manifested high convalescent-period IgG levels with a 4.6-fold drop at 1 year (Fig. 2b; Table 3, age 42). Two unvaccinated contacts exhibited very low, but detectable, anti-OPX IgG levels in convalescent-period samples (no 1-year samples were available) (Fig. 2d; Table 3, age 12 and age 13). One individual had detectable anti-OPX IgG and memory B-cell responses at 1 year (Fig. 2d and 4b; Table 3, 35-year-old). Though this individual was of an age to have received childhood smallpox vaccination, the person was reported to be unvaccinated, and the lack of detectable IgM or IgG at the first time point supports this.

Anti-OPX T-cell reactivity was also observed in some monkeypox contacts. One 39-year-old vaccinated contact displayed a conversion to CD4 positivity from convalescence to 1 year, albeit at low levels and with no detectable serological evidence of infection (Fig. 3a and b; Table 3). CD8 anti-OPX responses were not observed in unvaccinated contacts; however, two vaccinated contacts demonstrated elevated 1-year levels (Fig. 3c

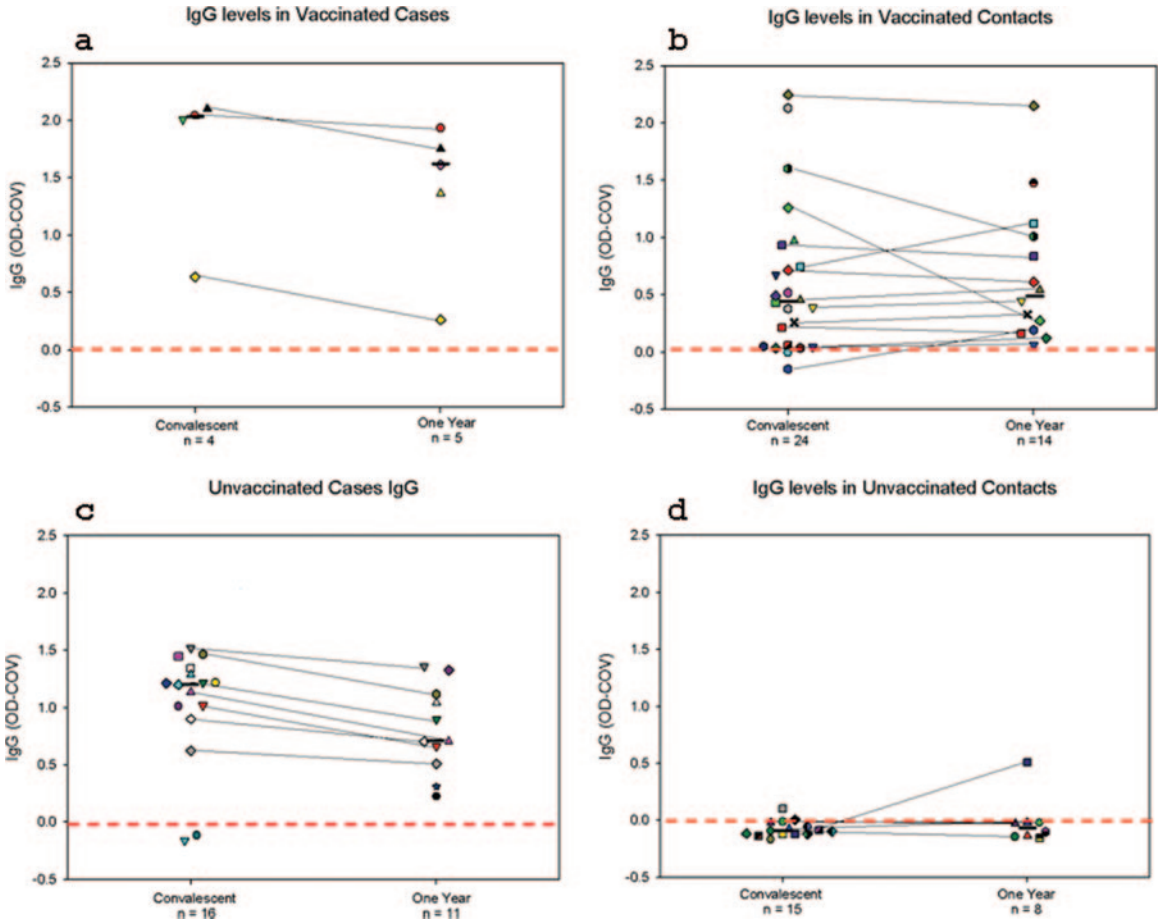

FIG. 2. ELISA of anti-OPX IgG responses in convalescent and 1-year sampling of all cases and contacts based on vaccination status. Results are presented as OD − COV where values above zero are considered positive. Symbols represent individuals involved in the study and remain consistent throughout the figures.

and d; Table 3, ages 37 and 56 years old). As described above, B-cell responses were also observed to change between convalescent and memory samplings in two vaccinated contacts (Fig. 4; Table 3).

Overall, in 11 contacts (Table 3), immune responses suggest some exposure to, and infection with, monkeypox. Most clearly, the presence of anti-OPX IgM (two contacts ages 49 and 60) or anti-OPX IgG at convalescent or 1-year time points (two unvaccinated contacts) suggests that monkeypox exposure resulted in infection, albeit without disease per the case definition. Similarly, changes in the level of reactivity of anti-OPX IgG, CD4, CD8, and/or B cells indicated antigenic boosting of immune response in some previously vaccinated individuals.

Immunologic characteristics of mild versus moderate-to-severe cases of human monkeypox. Descriptive differences in immune responses between mild and moderately/severely diseased cases in the U.S. outbreak are depicted in Fig. 5 and are based on those samples obtained within 4 weeks post-rash onset for antibody responses and at convalescence for cellular measures. Anti-OPX IgM responses are of greater magnitude for those with moderate/severe disease than for those with mild disease (Fig. 5a), while, at this time point, anti-OPX IgG responses are lower and less frequent within the moderate/severe disease group (Fig. 5b). Significant differences between anti-OPX CD4 and B-cell responses are not apparent (Fig. 5c and e), but there is a higher convalescent-period median anti-OPX CD8 response in those with moderate/severe disease (Fig. 5d).

Additionally, differences in the characteristics of mild versus moderate to severe monkeypox disease cases were analyzed, after categorizing high or low variables of immune response measurements, using the chi-square test and Fisher's exact test (Table 2). The absence of smallpox-vaccinated individuals in the younger age class made it impossible to control for age when evaluating the influence of vaccination on illness outcomes, and vice versa. However, moderate to severe disease was seen in 9 of 19 (47%) young cases (<31 years of age) compared to older cases (3 of 11 [27%]). Similarly, 10 of 23 (43.5%) unvaccinated cases experienced moderate to severe monkeypox illness compared to 1 of 6 (16.6%) vaccinated cases. Neither result achieved statistical significance (age, $P = 0.4425$; vaccination, $P = 0.3623$; Table 2).

High IgG titers were noted to be associated with mild illness (75% of moderate-to-severe cases had a low IgG response while a low IgG response was observed in only 46.15% of mild cases) although not in a statistically significant manner ($P = 0.37$, Table 2).

TABLE 3. Contacts showing immune marker(s) of potential infection

Age (yr)	Vaccination status	Convalescent-period sample value/1-yr sample value[a]					Exposure	Symptoms
		Serology		OPX-specific T cell		OPX-specific B cell		
		IgM	IgG	CD4	CD8			
12	No	−/NS	+/NS	−/NS	−/NS	NS/NS	Direct contact with prairie dog	None reported
13	No	−/NS	+/NS	−/NS	−/NS	NS/NS	Direct contact with prairie dog	None reported
35	No	−/−	−/+	NS/NS	NS/NS	NS/+	Indirect contact with prairie dog	None reported
37	Yes	−/−	−/+	+/+	−/+	−/+	Indirect contact with prairie dog	None reported
39	Yes	−/−	+/+	−/+	−/−	−/−	Direct contact with prairie dog	Fever, rash,[e] lymphadenopathy, headache
42	Yes	−/−	+/+	NS/NS	NS/NS	+/−	No contact with prairie dog (only with human case)	None reported
42	Yes	−/eq[b]	+(1.26)/+(0.274),[c] 4.6× drop	NS/NS	NS/NS	NS/−	Direct contact with prairie dog	Rash,[e] cough, sore throat, sweats, headache
46	Yes	−/−	+/NS	−/−	−/−	+(0.262)/+(0.02),[d] 13× drop	No contact with prairie dog (only with human case)	None reported
49	Yes	+/+	+/+	NS/NS	NS/NS	NS/+	Indirect contact with prairie dog	Fever, sore throat, sweats, chills, malaise, headache, diarrhea, myalgia, backache
56	Yes	−/−	+/+	−/−	−/+	+/NS	Indirect contact with prairie dog	None reported (multiple previous vaccinations for smallpox)
60	Yes	−/+	+/+	NS/NS	NS/NS	NS/−	Direct contact with prairie dog	None reported

[a] +, positive; −, negative; NS, no sample submitted.
[b] eq, equivocal result.
[c] Numerical values are ODs at 450 nm.
[d] Numerical values are the average OPX-specific IgG production B-cell spot divided by the total IgG spot count (all IgG-producing cells), equaling the frequency.
[e] Rash was reported; absence of fever and/or negative laboratory findings (PCR/culture/IgM) prevented a case classification.

The magnitude of vaccinia virus-specific cellular immunity to disease severity was also determined. No difference in disease severity was observed for those with higher OPX-specific CD4 counts versus lower OPX-specific CD4 counts (Table 2). High OPX-specific CD8 counts and low memory OPX-specific B-cell counts correlated in a nonsignificant manner with increased disease severity ($P = 0.62$ and $P = 0.387$, Table 2).

DISCUSSION

The 2003 outbreak in the United States represents the first opportunity to study human monkeypox outside Africa and is the largest case series of monkeypox infection with a monkeypox virus strain from the West African clade. Analysis of cases and contacts over a 1-year period allowed evaluation of the immune response and associations between disease, epidemiology, and remote vaccinia (smallpox) vaccine-derived immunity. The associations described provide new information regarding the nature of monkeypox infections, particularly pertaining to disease manifestation, immunity, and previous vaccination.

Analysis of anti-OPX immune responses in cases and contacts reveals specific findings regarding humoral and cellular immunity. Monkeypox cases most often had detectable anti-OPX IgM, IgG, and T- and B-cell immunity as well as aspects of disease consistent with the case definition. Also, while vaccinated contacts displayed signatures of vaccine immunity such as residual anti-OPX IgG and memory B cells, anti-OPX IgM and T-cell responses were absent or rare (Fig. 1 to 4; Table 3). For those contacts displaying IgM or T-cell responses, these aspects of immune detection suggest subclinical infection (Table 3). The presence of residual anti-OPX IgG in vaccinated contacts is consistent with other studies that demonstrate long-term persistence of anti-OPX IgG responses after vaccinia (smallpox) vaccination (4, 6, 8, 9, 12). However, anti-OPX IgM was observed only in monkeypox cases, with the exception of two vaccinated contacts (Table 3), supporting the use of this assay as a valuable epidemiologic tool for assessing monkeypox disease incidence. In the two cases where IgM was not detected, one was previously vaccinated and tested positive for IgM during the outbreak response (data not shown) and the other unvaccinated case also tested negative for IgM during the outbreak investigation. It is likely that the timing of the sample collection may have been outside the window for IgM detection.

In the present study, vaccinated contacts show waning CD8 T-cell responses, although previous studies indicate

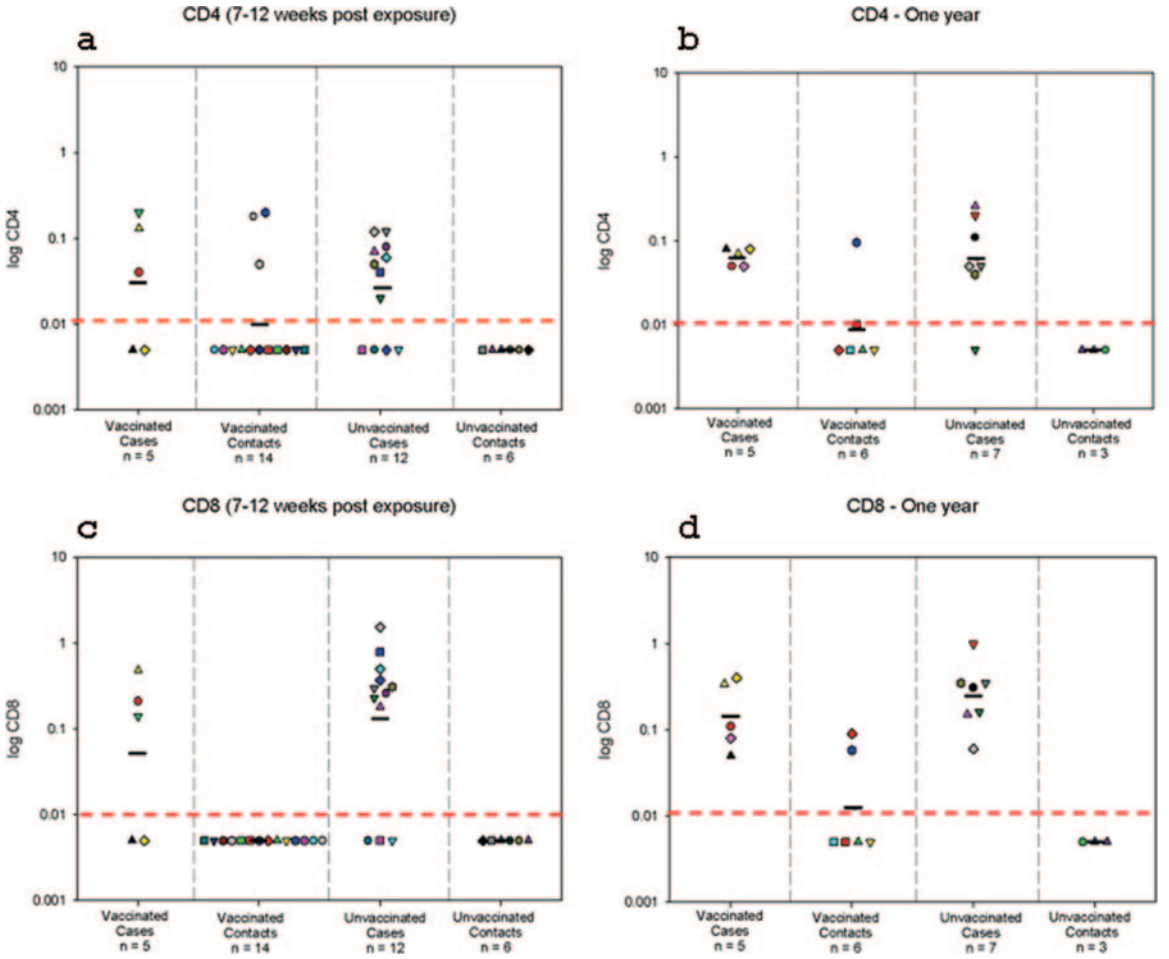

FIG. 3. Detection of OPX-specific T-cell memory responses in convalescent and 1-year sampling of cases and contacts. The limit of detection for this assay was 0.01% (red dashed line). Symbols represent individuals involved in the study and remain consistent throughout the figures.

that, while of low magnitude, residual OPX-specific CD8 responses are observed in some remote vaccinees years after immunization (1, 2, 7, 12, 18). The use of a single cell marker, in this study IFN-γ, may lessen sensitivity compared to multiple marker analysis. Based on these data, use of anti-OPX CD8 detection may provide another approach to evaluate for recent OPX infection in remotely vaccinated populations. Interestingly, two previously vaccinated contacts, without detectable OPX-specific CD8 responses at convalescent sampling, did develop responses at the 1-year measurement (Table 3); this may also represent exposure resultant in infection but not disease.

Evidence of subclinical infection was also observed in contacts by measure of serologic and cellular anti-OPX responses other than CD8 (Table 3). The detection of OPX-specific CD4 responses in three vaccinated contacts without rash illness, two at convalescent sampling and one of these individuals at 1 year (the other two did not provide 1-year samples), provides evidence of subclinical infection. Similarly, a drop in B-cell reactivity from convalescent to 1-year sampling was observed in two vaccinated contacts, with a rise for one vaccinated contact, indicating antigen exposure, expansion of memory B cells, and thus, recent OPX infection. However, given the presence of preexisting poxvirus immunity in much of the population, use of OPX-specific CD4 or B-cell detection as a diagnostic tool in vaccinated populations requires paired sampling to observe rises or falls in reactivity as an indication of recent infection.

Perhaps the most obvious question is whether remote smallpox vaccination affords protection from monkeypox infection. Previous studies with Congo Basin clade human monkeypox disease indicated that remote (childhood) smallpox vaccination diminished disease severity and provided 85% protection against acquisition of monkeypox disease in close family household contacts (14, 15). A later study in Central Africa in the 1990s ascribed an increase in disease incidence to the decrease in vaccine-derived protection due to increased time since vaccination campaigns and estimated an increase in household transmission (increased numbers of susceptible contacts) in the absence of vaccine-derived immunity (13). In the present study, 6 of 29 (24%) cases (confirmed $n = 4$, probable $n = 2$) had received previous (childhood) smallpox vaccination. Therefore, childhood vaccination does not provide adults with complete protection against monkeypox. Qualitatively, previous vaccination and high IgG levels appear to provide partial

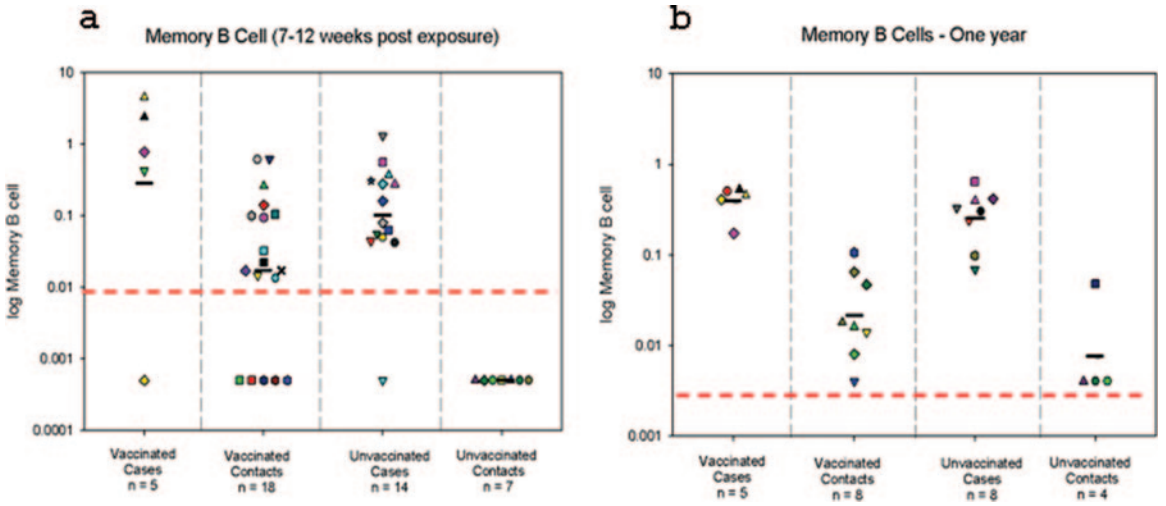

FIG. 4. Detection of OPX-specific B-cell memory responses in convalescent and 1-year sampling of cases and contacts. The limits of detection for these assays were 0.01% (a) and 0.004% (b) (dashed red line in both panels). Symbols represent individuals involved in the study and remain consistent throughout the figures.

protection. Disease severity is mitigated (Tables 1 and 2), albeit not to levels that were statistically significant; this could be due to a relatively small sample size. In household contacts of cases, the development of or changes in magnitude of immune responses to OPX antigen are supportive of subclinical infection that, in those previously vaccinated, could represent partial protection from disease (Table 3).

The absence of complete smallpox vaccine-derived protection during this outbreak is likely related to the longer interval in time from vaccination. The current outbreak occurred approximately 33 years post-vaccination cessation in the United States while the Congo Basin study in the 1980s was performed in African populations approximately 3 to 5 years post-vaccination cessation on that continent (at the level of individuals, vaccine was administered 32 to 41 years previously in the U.S. 2003 outbreak and 3 to 19 years previously in the Congo Basin study).

In addition to vaccination's effect on disease severity, the presence of immune markers of infection in some nonvaccinated contacts suggests that disease outcome could be dependent on the nature, dose, or route of viral exposure. Monkeypox virus infection during the U.S. outbreak resulted from animal, largely prairie dog (10, 21, 23), exposures/contacts that may differ from those associated with human monkeypox outbreaks in Central Africa. The route and dose of exposure, two factors that were not quantified in the present study, may also contribute to disease severity independently of vaccination history (Table 3) (22, 23). It is of note that two previously vaccinated household contacts in this study, with the only immune marker suggestive of recent anti-OPX exposure being B-cell immune response, did not recall contact with an infected animal and remembered only contact with another infected human case patient (Table 3, ages 42 and 49). Lastly, it is also possible that differences in the clinical presentation of the Western versus the Central African clades of virus may reflect differences in viral virulence and possibly different responses to waning vaccine-derived immunity (20).

In the absence of direct viral testing, differentiating between residual (vaccine-derived) immunity and recent-infection-induced immunity is imperative for diagnostic applications. While detection of anti-OPX IgG allows a measure of duration of immunity as well as prevalence, use of a long-lasting marker of infection (or vaccination) such as IgG fails to provide a compelling diagnostic tool in the absence of paired sera. Use of virus-specific IgM testing as a marker of infection provides an efficient and feasible diagnostic method independent of residual, vaccine-induced anti-OPX IgG, memory CD4, or B-cell responses, which may mask recent induction in these measures. Development of anti-OPX IgM responses in previously vaccinated cases also highlights significant differences in the antigenic makeup of vaccinia virus versus monkeypox virus to stimulate a secondary, heterologous IgM response that is absent in secondary vaccinia vaccinations (CDC, unpublished observation). In fact, identification of epitopes specific for monkeypox, or other OPXs, will drive the development of assays that are capable of discriminating immunity from different poxviruses (5, 11, 17).

Our findings from this study suggest that remote (30 years prior) vaccinia (smallpox) vaccination does not provide complete protection against systemic OPX infection (even against a relatively mild disease variant); in some cases, it may prevent systemic disease, but the relative contributions of infectious inoculum and route of exposure, in addition to remote vaccination, may significantly impact whether systemic illness, asymptomatic infection, or atypical illness manifests. Obtaining serologic and cellular samples early in infection and during acute illness may provide more complete information on the relationship of the immune response to disease (severity) outcomes. Future studies will investigate OPX species-specific epitopes and correlates of immunity which, in turn, will facilitate protective response analysis and species-specific diagnostic assay development. Our findings contribute data and a sample set for addressing vaccine and diagnostic issues impacting on vaccination strategies. Additionally, understanding differences in monkeypox

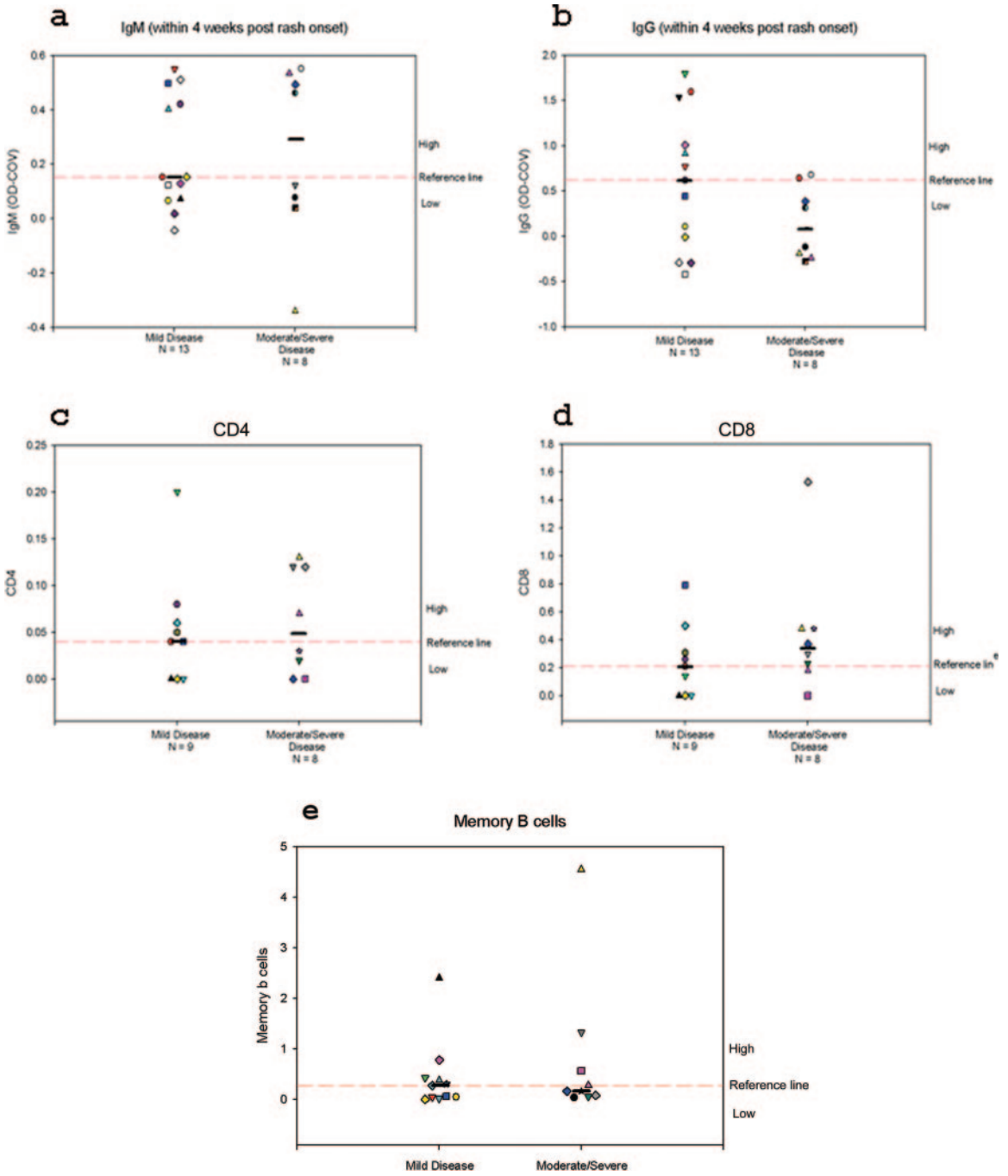

FIG. 5. Distribution of anti-OPX immune responses in monkeypox cases differentiated by disease severity as mild or moderate/severe, as described in Materials and Methods. (a) IgM (within 4 weeks post-rash onset); (b) IgG (4 weeks post-rash onset); (c) CD4 (convalescent sampling); (d) CD8 (convalescent sampling); (e) memory B cells (convalescent sampling). Symbols represent individuals involved in the study and remain consistent throughout the figures.

clade-specific human pathogenesis will further our understanding of protective host responses and vaccine-derived protection against monkeypox.

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